

The Listing of Claims

1. (Currently Amended) A methylotrophic recombinant yeast strain for producing a human insulin precursor, the strain having a genome comprising a copy of a first DNA construction and a copy of a second DNA construction, wherein said constructions ~~controlling~~are capable of directing the expression and secretion of a human insulin precursor, and wherein said DNA constructions ~~comprising~~comprise at least one DNA sequence encoding a human insulin precursor and ~~analogous~~analogs thereof.

2. (Currently Amended) The yeast strain of claim 1, wherein the strain ~~secreted~~secretes a human insulin precursor of the formula:

B(1-30)-Y1-Y2-A(1-21), wherein Y1 is lysine or arginine; Y2 is lysine or arginine; B(1-30) is the B peptide of the human insulin; and B(1-21) is the A peptide of human insulin.

3. (Currently Amended) The yeast strain of claim 1, wherein the strain is a member selected from *Hansenula*, *Pichia*, *Candida*, and *Torulopsis*.

4. (Currently Amended) The yeast strain of claim 3, wherein the strain is yeast *Pichia Pastorispastoris* deposited under accession number ATCC PTA-2260.

5. (Currently Amended) The yeast strain of claim 1, wherein the first DNA construction comprises:

a1) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region (promoter) operably linked to

b1) an exporting signal sequence ~~operable~~operably linked to

c1) a sequence encoding a human insulin precursor ~~operable~~operably linked to

d1) a 3' termination sequence linked to

e1) a selectable gene linked to

f1) a second ~~insertable~~insertion DNA sequence; and

the second DNA construction comprises:

a2) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region (promoter) operably linked to

b2) an exporting signal sequence ~~operable~~operably linked to

c2) a sequence encoding a human insulin precursor ~~operable~~operably linked to

d2) a 3' termination sequence linked to

e2) a selectable gene ~~distinct~~different from the selectable gene of the first DNA construction.

6. (Withdrawn - Currently Amended) The first DNA construction of claim 5, comprising at least one expression cassette for expressing the human insulin precursor, ~~the wherein said cassette comprising~~comprises:

a) a 5' regulatory region operably linked to

b) a DNA sequence encoding a signal sequence ~~operable~~operably linked to

c) a sequence encoding a human insulin precursor ~~operable~~operably linked to

d) a functional transcription termination sequence.

7. (Withdrawn) The first DNA construction of claim 5, wherein the selectable gene is HIS4.

8. (Withdrawn - Currently Amended) The first DNA construction of claim 5, wherein the 5' and 3' ends of said DNA construction comprises sequences ~~enough~~which are sufficiently homologous withto a target gene of the yeast to ~~permit~~allow for the replacement by a specific insertion of the DNA construction in ~~the~~a target gene, in the same relative orientation of the target gene in the yeast genome.

9. (Withdrawn) The first DNA construction of claim 5, wherein the 5' regulatory region is selected from the group consisting of the *Pichia pastoris* primary alcohol oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *Pichia pastoris* dihydroxyacetone synthase (DAS) gene promoter, the *Pichia pastoris* p40 regulatory regions promoter, the *Pichia pastoris* catalase promoter, and the glyceraldehyde dehydrogenase GAP promoter.

10. (Withdrawn) The first DNA construction of claim 5, wherein the signal sequence is the MF α of *Sacharomyces cerevisiae*.

11. (Withdrawn - Currently Amended) The first DNA construction of claim 5, wherein the functional transcription termination sequence is the termination sequence derived from *Pichia pastoris* AOX1 gene.

12. (Withdrawn - Currently Amended) The first DNA construction of claim 5, further comprising:

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region of *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) the sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3' termination sequence of *Pichia pastoris* AOX1 gene operably linked to

e) a *Pichia pastoris* HIS4 selection gene operably linked to

f) a second ~~insertable~~insertion DNA sequence corresponding to the *Pichia pastoris* AOX1 gene termination sequence.

13. (Withdrawn - Currently Amended) The first DNA construction of claim 5, wherein the sequence encoding the human insulin precursor is cloned in said construction

~~following~~adjacent to the protease site of the signal peptide, wherein all the secreted human insulin precursor contains, ~~in its amino terminal end,~~precursors contain the first amino acid of the human insulin precursor in the amino terminal end.

14. (Withdrawn) The first DNA construction of claims 5-13, wherein said DNA construction is incorporated into a vector selected from the group consisting of linear and circular vectors.

15. (Withdrawn - Currently Amended) The second DNA construction of claim 5, comprising at least one expression cassette for expressing the human insulin precursor, ~~the~~wherein said cassette comprising~~comprises~~:

- a) a 5' regulatory region operably linked to
- b) a DNA sequence encoding a signal sequence ~~operable~~operably linked to
- c) a sequence encoding a human insulin precursor ~~operable~~operably linked to
- d) a functional transcription termination sequence.

16. (Withdrawn - Currently Amended) The second DNA construction of claim 5, further comprising a selectable gene distinct from the selectable gene of the first DNA construction, wherein said selectable gene is the gene encoding zeocine resistance and wherein said selectable gene ~~permits to carry out~~allows for a second selection event.

17. (Withdrawn - Currently Amended) The second DNA construction of claim 5, wherein the 5' end of said insertion DNA construction comprises a single sequence ~~enough~~sufficiently homologous ~~with~~to a target gene of the yeast to ~~permit~~allow for the integration of the DNA construction in the target gene, in a single event.

18. (Withdrawn) The second DNA construction of claim 5, wherein the 5' regulatory region is selected from the group consisting of the *Pichia pastoris* primary alcohol

oxidase enzyme (AOX1) gene promoter, the secondary alcohol oxidase II enzyme (AOX2) gene promoter, the *Pichia pastoris* dihydroxyacetone synthase (DAS) gene promoter, the *Pichia pastoris* p40 regulatory regions promoter, the *Pichia pastoris* catalase promoter, and the glyceraldehyde dehydrogenate GAP promoter.

19. (Withdrawn) The second DNA construction of claim 5, wherein the signal sequence is MF α *Sacharomyces cerevisiae*.

20. (Withdrawn - Currently Amended) The second DNA construction of claim 5, wherein the functional transcription termination sequence is the termination sequence derived from the *Pichia pastoris* AOX1 gene.

21. (Withdrawn - Currently Amended) The second DNA construction of claim 5, further comprising:

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region of the *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) ~~the a~~ sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3' termination sequence of the *Pichia pastoris* AOX1 gene operably linked to

e) ~~the a~~ zeocine-resistant selection gene.

22. (Withdrawn - Currently Amended) The second DNA construction of claim 5, wherein the sequence encoding the human insulin precursor is cloned in said construction ~~following~~adjacent to the protease site, wherein all the secreted human insulin ~~precursor contains, in its amino terminal end,~~precursors contain the first amino acid of the human insulin precursor in the amino terminal end.

23. (Withdrawn) The second DNA construction of claims 15-22, wherein said DNA construction is incorporated into a vector selected from the group consisting of linear and circular vectors.

24. (Currently Amended) A method ~~offor~~ obtaining the yeast strain of claim 1, comprising the steps of:

i) transforming a yeast cell with a first DNA construction, ~~comprising: wherein said construction comprises:~~

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region of the *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* ~~operable~~operably linked to

c) ~~the-a~~ sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3' transcription termination sequence of the *Pichia pastoris* AOX1 gene operably linked to

e) a *Pichia pastoris* HIS4 selection gene operably linked to

f) a second ~~insertable~~insertion DNA sequence corresponding to the *Pichia pastoris* AOX1 gene termination sequence;

ii) selecting the yeast cells;

iii) isolating a yeast strain;

iv) re-transforming the yeast strain obtained in steps i)-iii) with a second DNA construction, ~~comprising: wherein said construction comprises:~~

a) a first ~~insertable~~insertion DNA sequence corresponding to a 5' regulatory region of the *Pichia pastoris* AOX1 gene operably linked to

b) the MF α signal sequence of *Sacharomyces cerevisiae* operably linked to

c) the sequence encoding the human insulin precursor of formula B(1-30)-Y1-Y2-A(1-21) ~~operable~~operably linked to

d) a 3' transcription termination sequence of the *Pichia pastoris* AOX1 gene operably linked to

e) ~~the~~ zeocine-resistant selection gene;

v) selecting the re-transformed yeast strain; and

vi) isolating the selected and re-transformed yeast strain.